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TITLE OF THE INVENTION (200 characters max)

Nanoparticulate Formulations of Magnetic Material and Bioactive Agents

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Respectfully submitted,

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Nanoparticulate Formulations of Magnetic Material and Bioactive Agents

FIELD OF THE INVENTION

The present invention is directed to the field of delivery of bioactive agents via nanoarticles comprising magnetic material, and the use thereof in therapeutic treatments.

BACKGROUND OF THE INVENTION

Chemotherapeutics are widely used in the treatment of cancer. While somewhat efficacious, the toxicity of the chemotherapeutics is severe and harmful, remission is often incomplete, and the eventual regrowth and spread of cancerous tissue is the norm. Efforts at localizing chemotherapeutics to the cancerous tissues, for instance through the attachment of chemotherapeutics to monoclonal antibodies which bind to receptors over-expressed on cancer cells, has thus far been a modest research success.

To reduce side effects, drug molecules have been directly conjugated to monoclonal antibodies (mAb). These constructs are known as immunoconjugates. For instance, Seattle Genetics (Seattle, WA) has a compound, SGN 15, which is a mAb that is functionalized with doxorubicin (dox) and binds to surface Le(y) antigens expressed in certain tumors. Despite improvement in biodistribution, immunoconjugates still struggle to deliver an effective dose to tumors and not to healthy tissue.

Hyperthermia, in which the temperature of cancerous tissue is raised, has demonstrated some efficacy in treating multiple types of cancer. One way that the temperature can be raised is through first localizing magnetic articles within a tumor, and subsequently heating the magnetic articles by subjecting them to an alternating magnetic field. Magnetic articles used for this type of therapeutic regimen may be made using several methods; one such method is described by Tan, et.al., in PCT application WO 01/88540 A1. It has been reported that certain magnetic particles can reach temperatures

in excess of 150 °C. However, to effectively heat the tumor mass, small particles require a high level of accumulation that is very difficult to achieve.

Cancer cells are under intrinsic oxidative stress and as such are vulnerable to free radical-induced apoptosis. The use of free radicals / free radical-producing agents as a therapeutic for the treatment of cancer has been investigated. For instance, hydrogen peroxide (H_2O_2) and superoxide anion ($O_2(-)$) are known to be involved in the cytotoxic action of a number of drugs (Akiyama and Natori, *Cancer Sci.*, 2003, 94: 400-404; Pelicano et al., *J Biol Chem.*, 2003, 278: 37832-9.) A further example is calicheamicin, an enediyne antibiotic capable of binding DNA which, following activation, results in cleavage of the double strand of the oligopyrimide-oligopurine sections of DNA. Recently, an immunoconjugate of this drug, gemtuzumab ozogamicin, has been approved by the FDA for the treatment of acute myelogenous leukemia. Thus, drug formulations that produce free radicals have demonstrated clear therapeutic benefit. If properly targeted to the tumor, the potential side effects of such formulations may be minimized.

Thus, although all the above cancer treatment methods are somewhat efficacious, improved cancer treatments are urgently needed.

SUMMARY OF THE INVENTION

This invention is directed to discrete articles, preferably nanoarticles, comprising magnetic material and a polymeric hydrogel scaffold at least partially surrounding the magnetic material.

In one embodiment of the invention, the nanoarticles further comprise bioactive agents (such as, for example, chemotherapeutics, cytotoxics, free radical-generating agents, other toxic agents and other therapeutic agents). The magnetic material and bioactive agent may be included in the same article, or each may be in a separate article and the two articles are placed in intimate relation to one another, preferably by covalent binding of the articles. Under the influence of an alternating or oscillating magnetic field, the temperature of the magnetic material and thus of the article may be raised through hysteretic heating. This heating results in the release of the bioactive agent from the article. For instance, antibiotics may be incorporated for the treatment of fungal and

bacterial infections, anti-inflammatory agents may be incorporated for the treatment of inflammatory diseases such as rheumatoid arthritis, and chemotherapeutic agents may be incorporated for the treatment of cancer.

In a second embodiment of the invention, the polymeric scaffold itself includes free radical-generating agents, the bonds of which break apart upon hysteretic heating to release free radicals. In this embodiment, bioactive agents may or may not also be attached to the nanoarticle.

In the case of chemotherapeutics, elevated temperature is known to increase the toxicity and anti-cancer potency of chemotherapeutics, so the localized heating generated by the magnetic material is expected to increase the potency of the released chemotherapeutic. While the heating itself is expected to result in the death of a portion of cancer cells in a tumor, heating of the entire tumor may not be achieved. However, the heating of the surrounding tissue is expected to aid in the diffusion of the chemotherapeutic into the tumor, resulting in the killing of more cancer cells. Outside of the applied magnetic field wherein the articles are heated, the articles of the invention will release the chemotherapeutic toxins only to a limited degree, such that the toxicity of the chemotherapeutic both systemically and in certain organs, such as in the heart, liver, kidney, and lung, is much reduced.

The articles of the invention may further comprise recognition elements that bind to certain biomolecules found in pathogenic tissue, such as certain cellular receptors that are overexpressed on the surface of some cancer cells.

The articles of the present invention may be used in the treatment of diseases such as cancer, inflammatory diseases, and infectious diseases. The articles of the present invention may also be used for magnetic resonance imaging (MRI). The invention is further directed to methods of synthesizing these nanoarticles.

DETAILED DESCRIPTION OF THE INVENTION

The terms "a" and "an" mean "one or more" when used herein.

By "water-soluble" is meant, herein and in the appended claims, having a solubility in water of greater that 10 mg/mL, and preferably greater than 50 mg/mL.

Disclosed herein are articles comprising magnetic material, a polymeric hydrogel scaffold at least partially surrounding the magnetic material, and, optionally, bioactive agents such as chemotherapeutic molecules, chemotherapeutic derivatives, free radical-releasing agents, or other toxic agents. Under the influence of an alternating magnetic field, the temperature of the article may be raised through hysteretic heating. This heating results in the release of the bioactive agent from the article and/or, where the nanoarticle includes free radical-generating components, the generation of free radicals. Additionally, as elevated temperature is known to increase the toxicity and anti-cancer potency of chemotherapeutics, the localized heating generated by the magnetic material can increase the potency of the released chemotherapeutic. While the heating itself is expected to result in the death of a portion of cancer cells in a tumor, heating of the entire tumor may not be achieved. However, the heating of the surrounding tissue is expected to aid in the diffusion of the chemotherapeutic or free radicals into the tumor, resulting in more complete cancer cell destruction. Outside of the applied magnetic field wherein the articles are heated, the articles of the invention will release the bioactive agents only to a limited degree, such that the toxicity of the bioactive agent both systemically and in certain organs, such as the heart, is much reduced.

The article of the invention may be further comprised of targeting agents or recognition elements that bind to certain biomolecules found in cancerous tissue, such as certain cellular receptors that are overexpressed on the surface of some cancer cells, including growth factor receptors, or in the tumor vasculature, such as integrins or growth factor receptors. Targeting agents that can be used include, but are not limited to, small molecules; vitamins, such as folate; peptides, such as those that target receptors; proteins, such as transferrin; and monoclonal antibodies and monoclonal antibody fragments. Unless otherwise specifically indicated, the terms "targeting agent" "recognition element" are used interchangeably herein.

While the articles of the invention may be larger in size, they are preferably from about 5 nm to about 1000 nm, more preferably from about 5 nm to about 500 nm, in diameter. Because of their size and hydrogel structure, the nanoarticles may circulate in the blood stream without being eliminated by the kidney or taken up by the RE system, and may localize in pathological tissue via passage through the pathological tissue's

leaky vasculature; the incorporation of targeting agents can further increase article accumulation in tissue to be treated, as described below.

Superparamagnetic articles, where the articles are magnetic only when a magnetic field is applied, are preferred. Preferred magnetic material for use in the invention disclosed herein are superparamagnetic materials such as magnetite, maghemite, and greigite. Magnetite may be incorporated into the articles in several ways.

Articles of the present invention may be made through the incorporation of bioactive agents, such as chemotherapeutic molecules or free radical-generating agents, to magnetite articles. Magnetic articles useful in the present invention include dextran-magnetite nanoparticles. These particles may be fabricated or may be purchased, for instance from companies such as Micromod Partikeltechnologie (Rostock, Germany). Magnetosomes from bacteria may also be used in the invention. In most magnetotactic bacteria, magnetosomes comprise membrane-bound crystals of magnetite, Fe_3O_4. Predominantly, members of the bacterial species *Magnetospirillum* form these highly ordered magnetite crystals to allow their orientation within the earth's magnetic field. The bacterial magnetosomes are characterized by narrow size distributions and uniform crystal habits, which are unknown from magnetite particles produced abiotically. Magnetosomes range in size from 35-100 nm and provide a large surface-to-volume ratio, which makes them attractive as carriers for the immobilization of pharmacologically active compounds. The magnetite crystals inside the magnetosome are of a size (below 50 nm) that renders them superparamagnetic

In one embodiment, magnetite articles are formed through a reverse microemulsion process as discussed below. Compared to the dextran magnetite particles which can be purchased, magnetite particles formed as discussed below can facilitate incorporation of free radical-generating agents, chemotherapeutics and recognition elements.

Article Scaffold Fabrication in Reverse Microemulsions: Components of the present invention can be fabricated using reverse microemulsions. In one embodiment, magnetic colloids are formed in the dispersed aqueous phase of a reverse microemulsion. Next, hydrogel scaffolds are formed around the magnetic core through the addition of

hydrophilic building blocks to the reverse microemulsion containing the magnetic colloids. The hydrophilic building blocks are then polymerized, forming a magnetic colloid-containing hydrogel nanoarticle. The organic solvent and non-reactive surfactants are removed after polymerization to yield crosslinked, water-soluble, magnetite-cored nanoscopic articles. Bioactive agents, if included in the final nanoarticle, may be incorporated either during or after hydrogel formation.

As used herein, the terms "nanoarticle scaffold", "hydrogel scaffold" and "scaffold" are used interchangeably and refer to the portion of the nanoarticle (the polymeric matrix structure) that incorporates the magnetic material.

Reverse microemulsions for magnetic colloid and scaffold fabrication are formed by combining aqueous buffer or water, building blocks, organic solvent, surfactants and initiators in the appropriate ratios to yield a stable phase of surfactant-stabilized aqueous nanodroplets dispersed in a continuous oil phase. Stable reverse microemulsion formulations can be found using known methods by those skilled in the art. They are discussed, for example, in *Microemulsion Systems*, edited by H. L. Rosano and M. Clausse, New York, N.Y., M. Dekker, 1987; and in *Handbook of Microemulsion Science and Technology*, edited by P. Kumar and K.L. Mittel, New York, N.Y., M. Dekker, 1999. In this invention, an aqueous phase with solubilized hydrophilic building blocks is added to an organic solvent containing one or more solubilized surfactants to form a reverse microemulsion.

The dispersed aqueous phase includes hydrophilic building blocks solubilized at about 5 to about 65 wt%, preferably about 5 to about 25 wt%, most preferably 10 to 20 wt%. While not wishing to be bound by theory, the use of high water-content hydrogel scaffolds also may reduce immunogenicity in end uses, because there is less foreign surface for immune system components to recognize. The high water content also provides compliancy through a more flexible scaffold. Thus, when attaching to cell surface receptors, the articles are able to conform to the cell surface, allowing more surface receptors to be bound. Binding more receptors may allow the article to better function as an antagonist. Additionally, while not wishing to be bound by theory, it is believed that article cell surface coverage can inhibit other cell signaling pathways.

Polymerization of the building blocks in the nanodroplets of the dispersed aqueous phase of the reverse microemulsion follows procedures known to those skilled in the art (see, for example, Odian G.G., *Principles of Polymerization, 3rd Ed.*, Wiley, New York, 1991; L.H. Sperling, *Introduction to Physical Polymer Science*, Chapter 1, pp. 1-21, John Wiley and Sons, New York, 1986; and R.B. Seymour and C.E. Carraher, *Polymer Chemistry*, Chapters 7-11, pp. 193-356, Dekker, New York, 1981). Polymerization has been performed in the dispersed phase of microemulsions and reverse microemulsions (for a review, see Antonietti, M.; and Basten, R., Macromol. *Chem. Phys.* 1995, 196, 441; for a study of the polymerization of a hydrophilic monomer in the dispersed aqueous phase of a reverse microemulsion, see Holtzscherer, C.; and Candau, F., *Colloids and Surfaces*, 1988, 29, 411). Such polymerization may yield articles in the 5 nm to 50 nm size range.

The size of the nanodroplets of the dispersed aqueous phase is determined by the relative amounts of water, surfactant and oil phases employed. Surfactants are utilized to stabilize the reverse microemulsion. These surfactants do not include crosslinkable moieties; they are not building blocks. Surfactants that may be used include commercially available surfactants such as Aerosol OT (AOT), polyethyleneoxy(n)nonylphenol (Igepal™, Rhodia Inc. Surfactants and Specialties, Cranbrook, NJ), sorbitan esters including sorbitan monooleate (Span® 80), sorbitan monolaurate (Span® 20), sorbitan monopalmitate (Span® 40), sorbitan monostearate (Span® 60), sorbitan trioleate (Span® 85), and sorbitan tristearate (Span® 65), which are available, for example, from Sigma (St Louis, MO). Sorbitan sesquioleate (Span® 83) is available from Aldrich Chemical Co., Inc. (Milwaukee, WI). Other surfactants that may be used include polyoxyethylenesorbitan (Tween®) compounds, including polyoxyethylenesorbitan monolaurate (Tween® 20 and Tween® 21), polyoxyethylenesorbitan monooleate (Tween® 80 and Tween® 80R), polyoxyethylenesorbitan monopalmitate (Tween® 40), polyoxyethylenesorbitan monostearate (Tween® 60 and Tween® 61), polyoxyethylenesorbitan trioleate (Tween® 85), and polyoxyethylenesorbitan tristearate (Tween® 65), which are available, for example, from Sigma (St Louis, MO). Other exemplary commercially available surfactants include polyethyleneoxy(40)-sorbitol hexaoleate ester (Atlas G-1086, ICI Specialties, Wilmington DE),

hexadecyltrimethylammonium bromide (CTAB, Aldrich), and linear alkylbenzene sulfonates (LAS, Ashland Chemical Co., Columbus, OH).

Other exemplary surfactants include fatty acid soaps, alkyl phosphates and dialkylphosphates, alkyl sulfates, alkyl sulfonates, primary amine salts, secondary amine salts, tertiary amine salts, quaternary amine salts, n-alkyl xanthates, n-alkyl ethoxylated sulfates, dialkyl sulfosuccinate salts, n-alkyl dimethyl betaines, n-alkyl phenyl polyoxyethylene ethers, n-alkyl polyoxyethylene ethers, sorbitan esters, polyethyleneoxy sorbitan esters, sorbitol esters and polyethyleneoxy sorbitol esters.

Other surfactants include lipids, such as phospholipids, glycolipids, cholesterol and cholesterol derivatives. Exemplary lipids include fatty acids or molecules comprising fatty acids, wherein the fatty acids include, for example, palmitate, oleate, laurate, myristate, stearate, arachidate, behenate, lignocerate, palmitoleate, linoleate, linolenate, and arachidonate, and salts thereof such as sodium salts. The fatty acids may be modified, for example, by conversion of the acid functionality to a sulfonate by a coupling reaction to a small molecule containing that moiety, or by other functional group conversions known to those skilled in the art.

Additionally, polyvinyl alcohol (PVA), polyvinylpirolidone (PVP), starch and their derivatives may find use as surfactants in the present invention.

Cationic lipids may be used as cosurfactants, such as cetyl trimethylammonium bromide/chloride (CTAB/CTAC), dioctadecyl dimethyl ammonium bromide/chloride (DODAB/DODAC), 1,2-diacyl-3-trimethylammonium propane (DOTAP), 1,2-diacyl-3-dimethyl ammonium propane (DODAP), [2,3-bis(oleoyl)propyl] trimethyl ammonium chloride (DOTMA), and [N-(N'-dimethylaminoethane)-carbamoyl]cholesterol, dioleoyl) (DC-Chol). Alcohols may also be used as cosurfactants, such as propanol, butanol, pentanol, hexanol, heptanol and octanol. Other alcohols with longer carbon chains may also be used.

Magnetic colloid formation: Magnetic cores, such as those comprised of magnetite or maghemite, can be formed using reverse microemulsions. For instance, Fe(II) is prepared by dissolving $FeSO_4 7H_2O$ in water and an Fe(III) solution is prepared by dissolving $FeCl_3 6H_2O$. These aqueous phases can then be combined in a reverse

microemulsion. Addition of a base results in the formation of a magnetite colloid. This colloid can then be incorporated into a hydrogel as described below. Prior to incorporation into the hydrogel, the magnetic colloid can be encapsulated in silica, and may be further functionalized via alkoxysilane derivatives to provide desired surface functionalites, such as acids and amines that can provide greater water solubility, covalent ligation to chemo-loaded articles, and facilitate hydrogel incorporation. Silica encapsulation is discussed by Grasset, et.al. in *European Cells and Materials*, Vol. 3, Suppl. 2, 2002, pp. 110-113.

Hydrogel formation: In one embodiment of the invention, a hydrogel that incorporates magnetic articles is formed. Hydrophilic building blocks with polymerizable groups are employed to form stable nanoarticle cores. In this embodiment, preferred building blocks are comprised of carbohydrate or derivatized carbohydrate. For example, the carbohydrate region may be derived from simple sugars, such as N-acetylglucosamine, N-acetylgalctosamine, N-acetylneuraminic acid, neuraminic acid, galacturonic acid, glucuronic acid, ioduronic acid, glucose, ribose, arabinose, xylose, lyxose, allose, altrose, apiose, mannose, gulose, idose, galactose, fucose, fructose, fructofuranose, rhamnose, arabinofuranose, and talose; a disaccharide, such as maltose, sucrose, lactose, or trehalose; a trisaccharide; a polysaccharide, such as cellulose, starch, glycogen, alginates, inulin, pullulan, dextran, dextran sulfate, chitosan, glycosaminoglycans, heparin, heparin sulfate, hyaluronates, tragacanth gums, xanthan, other carboxylic acid-containing carbohydrates, uronic acid-containing carbohydrates, lactulose, arabinogalactan, and their derivatives, and mixtures of any of these; or modified polysaccharides. Other representative carbohydrates include sorbitan, sorbitol, chitosan and glucosamine.

The carboxyl, amine and hydroxyl groups of the carbohydrates can be modified, or replaced, to include crosslinking groups, other functionalities, or combinations thereof.

Carbohydrate-based building blocks may be prepared from the carbohydrate precursor (e.g., sucrose, inulin, dextran, pullulan, etc.) by coupling technologies known in the art of bioorganic chemistry (see, for example, G Hermanson, *Bioconjugation Techniques*, Academic Press, San Diego, 1996, pp 27-40,155, 183-185, 615-617; and S.

Hanesian, *Preparative Carbohydrate Chemistry*, Marcel Dekker, New York, 1997). For example, a crosslinkable group may be attached to a carbohydrate via the dropwise addition of acryloyl chloride to an amine-functionalized sugar. Amine-functionalized sugars can be prepared by the reaction of ethylene diamine (or other amines) with 1,1'-carbonyldiimidazole-activated sugars. Ester-linked reactive groups can be synthesized through the reaction of acrylic or methacrylic anhydrides with the hydroxyl group of a carbohydrate such as inulin in pyridine. Aldehyde- and ketone-functionalized carbohydrates can be obtained by selective reduction of the sugar backbone or addition of a carbonyl-containing moiety. Other reactions that introduce an amine on the carbohydrate may also be used, many of which are outlined in *Bioconjugation Techniques (supra)*.

Carbohydrate-based building blocks may also be prepared by the partial (or complete) functionalization of the carbohydrate with moieties that are known to polymerize under free radical conditions. For example, methacrylic esters may be placed on a carbohydrate at varying substitution levels by the reaction of the carbohydrate with methacrylic anhydride or glycidyl methacrylate (Vervoort, L.; Van den Mooter, G.; Augustijins, P.; Kinget, R. *International Journal of Pharmaceutics,* 1998, 172, 127-135). Carbohydrate-based building blocks may also be prepared by chemoenzymatic methods (Martin, B. D. et. al., *Macromolecules*, 1992, 25, 7081), for example in which *Pseudomonas cepacia* catalyzes the transesterification of monosaccharides with vinyl acrylate in pyridine or by the direct addition of an acrylate (Piletsky, S., Andersson, H., Nicholls, *Macromolecules*, 1999, *32*, 633-636). Other functional groups may be present, as numerous derivatized carbohydrates are known to those familiar with the art of carbohydrate chemistry.

The carbohydrate structures are chosen in part for their hydrophilicity. Nano-articles that incorporate magnetic cores must possess highly hydrophilic scaffolds in order that high water solubility is maintained. Nanoarticles of the invention in one embodiment have a high water content for high water solubility. "High water content", as used herein, means an article comprised of about 65 to about 98 wt% water, more preferably about 75 to about 98 wt% water, and most preferably about 80 to 97 wt% water. Thus, the amount of breakdown products is less than articles with a higher

polymer concentration. The high water content scaffolds also can reduce immunogenicity, because there are fewer surfaces for immune system components to interact with.

Besides carbohydrate-based building blocks, other examples of acrylate- or acrylamide-derivatized polymeric building blocks include polyethylene glycol-based molecules, such as polyethyleneglycol multiacrylates of molecular weights ranging from 200 to 10,000 daltons.

In one embodiment of the invention, to facilitate metabolism of the hydrogel scaffold, degradable linkages are included within the crosslinked scaffold. Degradable linkages can be included through the use of polylactide, polyglycolide, poly(lactide-co-glycolide), polyphosphazine, polyposphate, polycarbonate, polyamino acid, polyanhydride, and polyorthoester – based building blocks, among others. Additionally, degradable linkages may be used to attach polymerizable moieties to carbohydrates. For instance, IMMA contains ester moieties that connect the inulin carbohydrate backbone to the alkyl chain that is formed upon free radical polymerization used to generate the scaffold of the present invention. Additionally, small molecule crosslinking agents containing similar hydrolyzable moieties as the polymers such as carbonates, esters, urethanes, orthoesters, amides, imides, imidoxy, hydrazides, thiocarbazides, and phosphates may be used as building blocks. To function as degradable components in the hydrogel scaffold, these building blocks must be functionalized with two or more polymerizable moieties. For example, polyglycolide diacrylate, polyorthoester diacrylate and acrylate-substituted polyphosphazine, acrylate-substituted polyamino acid, or acrylate-substituted polyphosphate polymers can be used as degradable building blocks.

Methacrylate or acrylamide moieties can be employed instead of acrylate moieties in the above examples. Similarly, small molecules containing a hydrolyzable segment and two or more acrylates, methacrylates, or acrylamides may be used. Such degradable polymers and small molecule building blocks may be functionalized with acrylate, methacrylate, acrylamide or similar moieties by methods known in the art.

The nanoarticle scaffolds and the scaffold breakdown products of this invention are designed to be non-toxic and eliminated from the body. They may have degradable, preferably carbohydrate-based, polyamino acid-based, polyester-based, or PEG-based

cores, with the rate of degradation controlled by the identity of the sugar, crosslink density, and other features. Thus, the articles can be metabolized in the body, preventing undesirable accumulation in the body.

Incorporation of Bioactive Agent Molecules into Articles: The terms "drug", "drug-conjugate", "bioactive agent" and "therapeutic agent" are used interchangeably herein. In one preferred embodiment, free radical-generating agents are covalently linked to the article scaffold. In another preferred embodiment, drug molecules are covalently linked to the article scaffold. In a presently preferred embodiment, drugs that may be advantageously employed in the present invention include, but are not limited to, chemotherapeutics such as doxorubicin, paclitaxel, gemcitibine, vincristine, cisplatin, carboplatin, chlorambucil, topotecan, methotrexate, derivatives of these compounds, and any other FDA-approved chemotherapeutic, as well as molecules that may act as chemotherapeutics but that are not yet commercialized, and derivatives and analogues of all of the above chemotherapeutics.

For example, the chemotherapeutic doxorubicin may be attached to the scaffold through a EDC coupling reaction between the amine moiety on doxorubicin and a carboxylic acid moiety included in the hydrogel scaffold, for example by using sodium acrylate (NaA), malonate acrylamide (MalAc) or CiBA as a building block. In another embodiment, doxorubicin may be attached via an imine bond by reacting doxorubicin's amine moiety with an aldehyde moiety of the hydrogel scaffold. An aldehyde may be created by first using a carbohydrate-based building block to form the article, and then oxidizing the carbohydrate after the article is formed. In another embodiment, doxorubicin may be attached to the article matrix through its ketone moiety.

Carbohydrazide or other dihydrazide or di-amino-oxy functionalized structures may be used to link doxorubicin to a scaffold that contains an aldehyde or ketone through the formation of a hydrazone bond. An aldehyde or ketone may be incorporated into the scaffold through the use of a ketone-containing acrylate building block such as DAA. A hydrazone bond may favorably release the therapeutic compound under the mildly acidic physiological conditions encountered upon article endocytosis and entrance into lysosomes.

In another embodiment, nanoarticle scaffolds comprised of amino groups, for example through the inclusion of APMA or methacrylate-functionalized short peptide (prepared according to US patent 5,037,883) building blocks, may be used to covalently attach cyclosporins that contain carboxylate linkages. Cyclosporin drugs may find applications for pathologies that benefit from immunosuppression, such as inflammatory diseases, and for organ transplantation.

In another embodiment, nanoarticle scaffolds comprised of aldehyde or ketone groups (for example incorporated through the inclusion of DAA, levulinic acrylamide or oxidized carboxylates such as inulin or dextran building blocks) may be used to covalently attach drugs or drug derivatives that contain a moiety, for instance calicheamicin, through the use of a hydrazone coupling scheme. This coupling scheme results in a hydrazone bond that is hydrolytically labile, especially at low pH found in lysosomes (Bernstein I., et al., *Bioconjugate Chem.*, 2002, 13, 40-46).

In another embodiment, nano-article scaffolds comprised of acid or anhydride groups, for example incorporated through the inclusion of sodium acrylate or anhydride building blocks, may be used to covalently attach dexamethasone, through the use of an amide coupling scheme.

Nanoarticle scaffolds comprised of carboxylate groups (for example, incorporated through the inclusion of sodium acrylate (NaA), CiBA or MAIAc building blocks) may be used to covalently attach drugs or drug derivatives that contain an amine moiety, for instance peptide-modified camptothecin (Frigerio E., et al., *J. Controlled Release*, 2000, 65, 105-119) through an EDC-NHS coupling scheme.

In another embodiment, when the nanoarticle scaffolds are comprised of aldehyde or ketone groups (which may be incorporated through the use of DAA, levulinic acrylamide or oxidized carboxylates such as inulin or dextran), a drug or drug derivative possessing an amine, such as gemcitabine, may be incorporated through the use of a "Schiff base" coupling scheme. The imide bond formed from the attachment of gemcitabine to DAA can be cleaved in acidic media. During internalization, the drug is taken up by the cell, where it is exposed to the acidic environment of the lysosome, thereby releasing gemcitabine in its unmodified form.

In another embodiment, nanoarticle scaffolds comprised of carboxyl groups (for example, incorporated through the inclusion of CiBA, MalAc or NaA building blocks) may be used to covalently attach drugs or drug derivatives that contain a moiety, for instance salicylic acid, through the use of an EDC-NHS coupling scheme. For instance, the hydroxyl group of salicylic acid will react with the carboxyl group of the CiBA, MalAc or NaA to form an ester link. Hydrolysis or the enzyme esterase will cleave the ester bond between salicylic acid and the carboxylic acid groups of CiBA or NaA, releasing salicylic acid in an unmodified form.

In another embodiment, a drug structure may be modified to facilitate attachment to a nanoarticle scaffold. For instance, the 2'-hydroxyl group of paclitaxel can be reacted with multiple linkers that enable the coupling to nanoarticle scaffolds. For example, the acid moiety of a resin-immobilized glycine linker can be attached to paclitaxel using a carbodiimide; the resulting compound can be cleaved at the site of the amine using 1% TFA, producing a free amine which can be conjugated with nano-articles possessing carboxylates using an EDC coupling scheme.

In another embodiment, paclitaxel-2'-succinate (Deutsch H., et al., *J Med. Chem.*, 1989, 32, 788-792) conjugation to the nanoarticle is possible using a carbodiimide-mediated amide coupling. This coupling occurs between the paclitaxel-2'-succinate group and an amine group of the APMA component of the nano-article to form a labile ester.

In another embodiment, the nanoarticle can be directly coupled to paclitaxel by reacting the acid-functionalized (NaA) nanoarticle to the 2'-hydroxyl group of paclitaxel. This chemical pathway has been previously described using a poly(L-glutamic acid)-paclitaxel conjugate (Li H., et al., Cancer Res., 1998, 58, 2404-2409).

In another embodiment, nanoarticle scaffolds containing carboxylic acids, for example incorporated through the inclusion of sodium acrylate (NaA) building blocks, may be used to covalently attach drugs or drug derivatives that contain a moiety, for instance 5-flourouracil (5FU) (or derivatives) through the use of an amide forming coupling reaction between an amine-functionalized 5FU derivative and the carboxylic acids located on the nanoarticle. The synthesis of 1-alkylcarbonyloxymethyl derivatives of 5FU has been previously described and those materials have been demonstrated to

release 5FU in an unmodified form (Taylor H.E.; Sloan K.B., *Journal of Pharmaceutical Sciences*, 1998, 87, 15). The application of this synthetic route will yield the necessary amine-functionalized 5FU, whilst realizing a similar release profile.

Nanoarticle scaffolds comprised of acid groups (for example incorporated through the inclusion of CiBA, MalAc and NaA building blocks) may be used to covalently attach drugs or drug derivatives that contain a carboxylate moiety, for instance methotrexate, by first coupling the drug or drug derivative to boc-protected ethanolamine to form an ester, and then coupling to the nanoarticle through an EDC coupling scheme after deprotecting the modified drug. This ester conjugate is known to hydrolyze at low pH, releasing the drug in its original form (Wilson J.M., et al., *Biochem Biophys. Res. Commun.*, 1992, 184, 300-305; Ohkuma S., Poole B., *Proc. Natl. Acad. Sci. USA*, 1978, 75, 3327-3331). Such conditions of low pH are found in cellular lysosomes. These nanoarticles may find use in the treatment of multiple pathologies, including cancer and inflammatory conditions such as rheumatoid arthritis and inflammatory bowel disease.

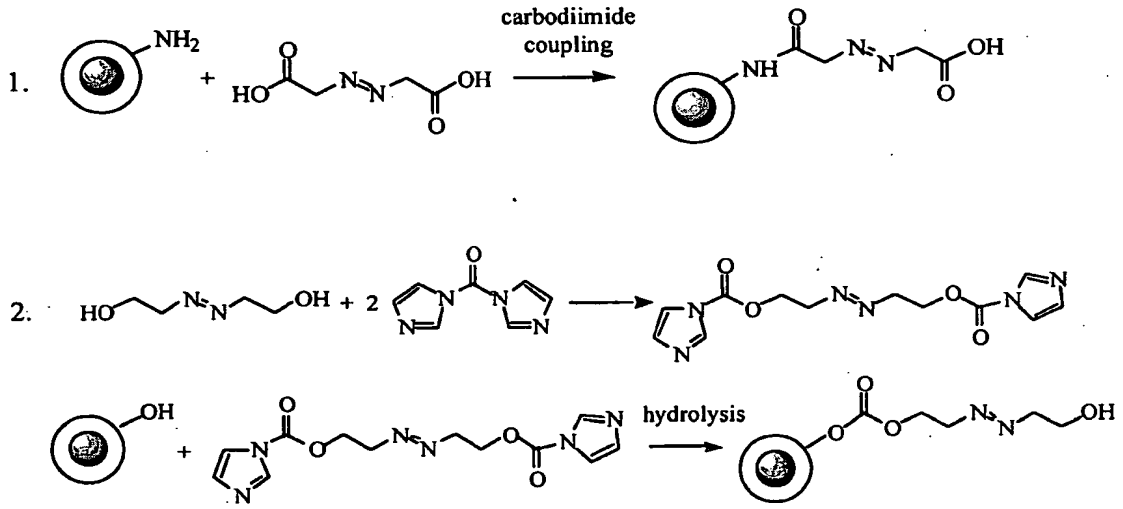
In another embodiment, platinum conjugates are incorporated onto nanoarticle scaffolds. The platinum may be in the IInd or IVth oxidation state. There remains a distinct need for new platinum chelates with further improvements in therapeutic index compared with the currently-approved platinum chelates. Such chelates should be highly water-soluble and stable in an aqueous environment, but sufficiently labile in tumor cells to provide species capable of crosslinking DNA and ultimately causing tumor cell death. The hydrogel network of the nanoarticles of the present invention combined with the capacity to fine tune its chemical composition allows for such a high water solubility and flexibility in the way to attach chelates, which constitute a significant improvement over WO 9847537. It has been shown that changes in the platinum chelate structure could modify the spectrum of tumor types for which platinum therapy is effective and/or alter the toxicity profile of the chelate. In one important embodiment of the invention the platinum is complexed to the hydrogel matrix via O,N-ligation, which is expected to yield a more stable compound.

This can be accomplished preferentially for nanoarticles obtained by free-radical polymerization, and containing a combination of acid functions (such as from NaA) and amines (such as from APMA) or amides moieties (such as acrylamide), or building blocks

carrying both types of functions such as CIBA, MalAc or methacryloylate-functionalized short peptides made according to US 5,037,883. Such moieties provide attachment points to generate a O,N-cis platinum nanoarticle conjugate, and also allow the possibility of targeting the nanoarticle. The high toxicity of platinum derivatives renders the specific targeting of the nanoarticles to organs or tumor sites greatly beneficial.

Hydrogel-coated magnetic nanoarticles that degrade to form radicals can be synthesized by two synthetic strategies. In the first strategy, radical-generating functionalities are attached to the hydrogel surrounding a nanoarticle (Figure 1).

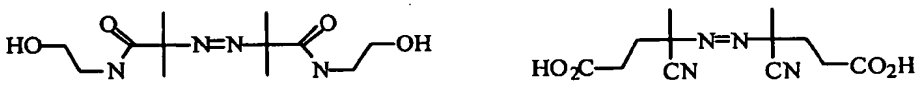
Figure 1. Two synthetic routes which could be used to attach a radical generating species to a hydrogel coated nanoparticle.



With the variety of temperature sensitivities that radical sources can display (see, e.g., Odian, G. *Principles of Polymerization* 2nd Edition, Wiley-Interscience, 1981, 196), a radical-producing structure that would be satisfactory for the temperature profile of the target product would be selected. Radical-generating functionalities, such as azo or peroxides, can be attached to the hydrogel through a variety of chemical bonds such as amides or carbonates. The exact strategy used would depend on the properties of the radical initiator being attached. For example, the use of peroxide radical sources would

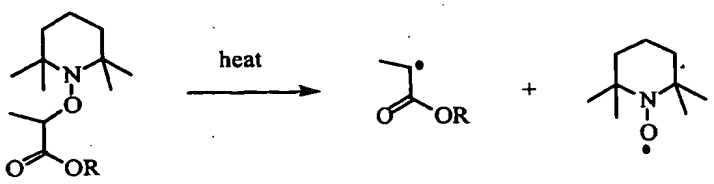
preclude the use of amine-containing hydrogels, because of the increased instability of those compounds in the presence of an amine. However, azo-containing compounds would be generally useful in either proposed attachment method. Two commercially available radical generating compounds have been identified (Figure 2).

Figure 2. Two commercially available azo compounds.



A second strategy in which the hydrogel coating of the nanoparticle degrades to form radical species is also encompassed by the invention. In this strategy, the unique reactivity of alkoxyamine compounds to generate radical species is used (for a general discussion of alkoxy amines, their thermal behavior, stability, and use in polymer chemistry, see Lizotte, J., "Synthesis and Characterization of Tailored Macromolecules via Stable Free Radical Polymerization Methodologies", *Dissertation Virginia Polytechnic Institute and State University*, 2003, Chapter 2) (Figure 3).

Figure 3. On heating, alkoxyamine compounds degrade to radical species. In this nonspecific example, the stable nitroxyl compound TEMPO is released.



A magnetic nanoparticle can be coated with an alkoxy amine-containing hydrogel by a strategy similar to that described above, using reverse microemulsions. In this procedure, a 2,2,6,6-tetramethyl-1-piperdinyloxy (TEMPO)-derivatized polysaccharide, radical initiator, acrylate-functionalized poly(ethylene glycol), and acrylic acid monomer are dissolved in the aqueous phase of the magnetic nanoarticle-containing reverse

microemulsion. Unlike typical polymerizations, the radical initiator would have a stoichiometry close to that of the total acrylate concentration of the monomers. On photolysis, the radical initiators initiate polymerization of the monomer species. However, the high concentration of the TEMPO nitroxyl (equal to that of the total acrylate concentration) would quickly react with the monomer-centered radical, making the stable alkoxy amine (Figure 4, Figure 5).

Figure 4. A representative reaction in which TEMPO is used to make a stable alkoxyamine (Hawker, C. et al., *Macromolecules*, 1996, 29, 5245). The alkoxyamine product, on heating, generates radical species, which will initiate a polymerization reaction.

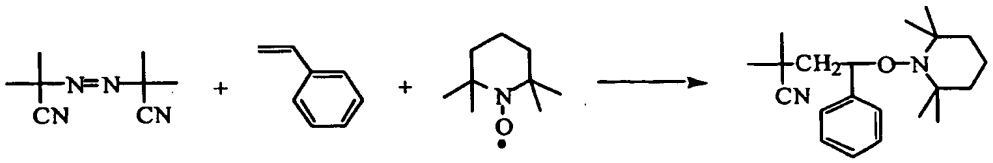
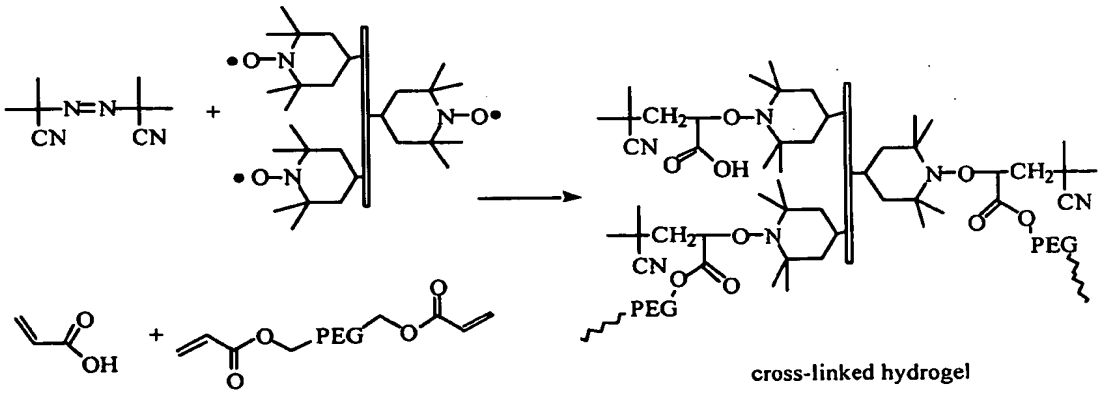


Figure 5. The reaction of a mixture containing a radical initiator, TEMPO-derivatized carbohydrate, acrylic acid, and PEG diacrylate. This reaction, if done in a reverse microemulsion containing a magnetic nanoarticle, will make a hydrogel-coated nanoarticle.



The alkoxyamine-containing nanoarticles, when heated, will degrade, releasing radicals.

Incorporation of Recognition Elements: After the assembled building blocks are crosslinked to form the hydrogel scaffold and the bioactive agent has been covalently attached to the scaffold, the article surface may be functionalized with recognition elements ("REs"). The REs can target a multitude of disease-associated biomolecules. Tumor-associated targets include folate receptors, transferrin receptors, erbB1, erbB2, erbB3, erbB4, CMET, CEA, EphA2, carcinoembryonic (CEA) antigen, mucin antigens, including Muc-1, cellular adhesion, of the cluster differentiation (CD) antigen family. Vascular targets associated with multiple pathologies, including cancer, include VEGFR-1, VEGFR-2, and integrins, including integrin $\alpha v\beta 3$, and integrin $\alpha v\beta 1$. Additional targets are extracellular proteins such as matrix metalloproteinases (MMPs), the collagen family, and fibrin.

The REs can be linked either directly or through a linker molecule to the surface of the nanoarticle. In a linker configuration, part or all of the REs are "displayed" at the end terminus of the tether. Therefore, in one application of the invention, the articles consist of REs displayed on a hydrogel scaffold. In another embodiment of the invention, the articles consist of an RE, such as a high affinity peptide, linked to the surface of the article core scaffold via a linker molecule, the linker comprising, in a preferred embodiment, polyethylene glycol (PEG). The PEG linker can be linear with reactive functionalities at both of the chain terminals; the PEG linker can also be multi-armed, for instance possessing three, four, five, six, eight arms or more, with two or more of the arms possessing reactive functionalities that can be used to attach the PEG to the nanoarticle scaffold and the RE to the PEG.

For each of these embodiments, it is possible to functionalize the articles with several coupling strategies, varying both the order of addition of the different components and the reactive chemical moieties used for the coupling.

The components may be attached to one another in the following sequences. The hydrogel scaffold is first reacted with a di-functional PEG-containing tether, followed by functionalization of the free terminus of a portion of the PEG chain with a RE. Alternatively, the RE is coupled first to the PEG-containing tether, followed by the attachment of the other PEG terminus to the scaffold.

Several combinations of reactive moieties can be chosen to attach the RE to the tether and to attach the tether to the nanoarticle scaffold. In using a series of orthogonal reaction sets, varying some of the scaffold building blocks and/or tethering arms, it is also possible to attach REs with different molecular structures that bind to different receptors, onto the same article scaffold in well-controlled proportions. Reactions using orthogonal reactive pairs can be done simultaneously or sequentially.

It is preferable to functionalize the articles in an aqueous system. The surfactants and the oil phase, residual from the synthesis of the hydrogel scaffold, can be removed through the use (singularly or in combination) of solvent washing, for instance using ethanol to solubilize the surfactant and oil while precipitating the articles; surfactant-adsorbing beads; dialysis; or the use of aqueous systems such as 4M urea. Methods for surfactant removal are known in the art.

The RE must contain a functionality that allows its attachment to the article. Preferentially, although not necessarily, this functionality is one member of a pair of chemoselective reagents selected to aid the coupling reaction (Lemieux, G., Bertozzi, C., *Trends in Biotechnology*, 1998, 16, 506-513). For example, when the article surface (and/or linkers grafted to its surface) displays a halo acetal, a peptide RE may be attached through a sulfhydryl moiety. A sulfhydryl moiety in the RE structure can be accomplished through inclusion of a cysteine residue.

Coupling is also possible between a primary amine on the article or the linker terminus and a carboxylic acid on the RE. A carboxylate in the peptide structure can be found either on its terminal amino acid, for linear peptides, or through the inclusion of aspartic or glutamic acid residues. The opposite configuration, where the carboxylic acid is on the article and a primary amine belongs to the peptide, is also easily accessible. Many polymerizable building blocks contain acidic moieties, which are accessible at the surface of the beads after their polymerization. As for poly(amino acid)-based REs, a primary amine function can be found either at its N-terminus (if it is linear) and/or via introduction of a lysine residue.

Another example of reactive chemical pairs consists of the coupling of a sulfhydryl with a halo acetal or maleimide moiety. The maleimide function can be easily introduced, either on a peptide, a linker, or the surface of the articles, by reacting other common

functionalities (such as carboxylic acids, amines, thiols or alcohols) with linkers through methods known to one of skill in the art, such as described for example by G. T. Hermanson in *Bioconjugate Techniques*, Academic Press Ed., 1996. In a preferred embodiment, the inclusion of CiBA or other disulfide-containing building blocks, in the scaffold facilitates the attachment of REs through thiol reactive moieties. After scaffold formation, reduction of the disulfide linkage in CiBA produces free thiols. Linker molecules containing groups that are reactive with thiol, such as bromoacetamide or maleimide, are added to the reduced therapeutic agent-containing article to attach the linker to the article scaffold. REs are then added, which react with the free terminus of the linker molecules to give RE-functionalized articles. Alternatively, the RE may be attached to one end of the linker molecule prior to attachment of the linker molecule to the reduced article.

Peptides can also be coupled to the article and/or the tether with a reaction between an amino-oxy function and an aldehyde or ketone moiety. The amino-oxy moiety (either on the articles or in the peptide) can be introduced, starting from other common functionalities (such as amines for example), by a series of transformations known to those skilled in the art. In the same way, aldehyde- or ketone-containing articles and aldehyde-containing peptides are readily synthesized by known methods.

The resulting RE-functionalized, bioactive agent-containing articles may be used immediately, may be stored as a liquid solution, or may be lyophilized for long-term storage.

The REs may be any small or large molecular structure that provides the desired binding interaction(s) with the cell surface receptors of the targeted molecule. The number of recognition element moieties per article can range from 2 to about 1000, preferably from 2 to 500, and most preferably from 2 to 100. The articles may optionally further be comprised of more than one type of RE. As used herein, a RE "type" is defined as a specific molecular structure.

In one embodiment REs are comprised of peptides. Peptides used as REs according to this invention will generally possess dissociation constants between 10^{-4} and 10^{-9} M or lower. Such REs may be comprised of known peptide ligands. For instance, Phoenix Peptides' peptide ligand-receptor library

(http://www.phoenixpeptide.com/Peptidelibrarylist.htm) contains thousands of known peptide ligands to receptors of potential therapeutic value. The peptides may be natural peptides such as, for example, lactams, dalargin and other enkaphalins, endorphins, angiotensin II, gonadotropin releasing hormone, melanocyte-stimulating hormone, thrombin receptor fragment, myelin, and antigenic peptides. Peptide building blocks useful in this invention may be discovered via high throughput screening of peptide libraries (e.g. phage display libraries or libraries of linear sequences displayed on beads) to a protein of interest. Such screening methods are known in the art (for example, see C.F. Barbas, D. R. Burton, J. K. Scott, G. J. Silverman, *Phage Display*, 2001, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY). The high affinity peptides may be comprised of naturally-occurring amino acids, modified amino acids or completely synthetic amino acids. The length of the recognition portion of the peptide can vary from about 3 to about 100 amino acids. Preferably, the recognition portion of the peptide ranges from about 3 to about 15 amino acids, and more preferably from 3 to 10 amino acids. Shorter sequences are preferred because peptides of less than 15 amino acids may be less immunogenic compared to longer peptide sequences. Small peptides have the additional advantage that their libraries can be rapidly screened. Also, they may be more easily synthesized using solid-state techniques.

Particular peptides of interest are comprised of the amino acid sequence YCPIWKFPDEECY, or other sequences found in Greene, et.al., *J. Biol. Chem.*, 2002, 277(31), 28330-28339, that bind to erbB1; peptides comprised of the amino acid sequence CdFCDGFdYACYMDV, where dF and dY representing the D isomer of the amino acid residues or other sequences delineated in Murali, *J. Med. Chem.*, 2001, 44, 2565 - 2574, as REs; peptides disclosed in PCT WO 01/74849 that bind to CEA; and peptides comprised of the amino acid sequence ATWLPPR, as described in Demangel, et.al., *EMBO J.*, 2000, 19(7), 1525-1533.

REs may be comprised of a variety of other molecular structures, including vitamins such as folate, growth factors such as EGF, proteins such as transferrin, antibodies, antibody fragments, lectins, nucleic acids, and other receptor ligands. Humanized or fully human antibodies, and humanized or fully human antibody fragments are preferred for use in the present invention.

Additionally, it will be possible to design other non-protein compounds to be employed as the binding moiety, using techniques known to those working in the area of drug design. Such methods include, but are not limited to, self-consistent field (SCF) analysis, configuration interaction (CI) analysis, and normal mode dynamics computer programs, all of which are well described in the scientific literature. See, Rein et al., *Computer-Assisted Modeling of Receptor-Ligand Interactions*, Alan Liss, New York (1989). Preparation of non-protein compounds and moieties will depend on their structure and other characteristics and may normally be achieved by standard chemical synthesis techniques. See, for example, *Methods in Carbohydrate Chemistry, Vols. I-VII*; Analysis and Preparation of Sugars, Whistler et al., Eds., Academic Press, Inc., Orlando (1962), the disclosures of which are incorporated herein by reference.

The use of multiple RE molecules of the same molecular structure or of different molecular structure to make up the article can increase the avidity of the article. As used in the present invention, "high affinity" means a binding of a single RE to a single target molecule with a binding constant stronger than 10^{-4} M, while "avidity" means the binding of two or more such RE units to two or more target molecules on a cell or molecular complex.

Composite Article Assembly: Articles of the present invention may be made through the attachment of one or more nanoarticles comprised of bioactive agents, such as a chemotherapeutic or a free radical-generating agent, to one or more nanoarticles comprised of magnetic material. For example, nanoarticles comprised of bioactive molecules may be made through microemulsion polymerization schemes detailed above or may be purchased through commercial sources. Similarly, nanoarticles comprised of magnetite may be made through a microemulsion fabrication scheme also detailed above or may be purchased from commercial sources. These two types of articles, one containing magnetite and one containing bioactive agents, can then be attached to one another to form the articles of the invention.

Chemotherapeutic-containing articles useful in this embodiment include liposomal-encapsulated doxorubicin. To prepare for magnetic article attachment, a functionalized PEG-lipid, such as a maleimide-functionalized PEG-lipid derivative, such as DSPE-

PEG(2000)Maleimide (commercially obtainable from sources such as Avanti Polar Lipids, Inc.), is incorporated into the a liposome-encapsulated doxorubicin, such as Doxil, a commercialized liposomal doxorubicin product. While not wishing to be bound by theory, it is believed that the heating of the magnetic material can release the encapsulated chemotherapeutic by causing a phase transition of the lipid bilayer and also through the accelerated dissolution of the crystalline doxorubicin in the aqueous core of the liposomes.

One embodiment of the invention uses liposomes that are surface-functionalized with aminodextran. Free amines may be converted to thiols with Traut's reagent, which can then be reacted with moieties such as maleimides or halo-acetals on previously-formed magnetic articles.

In one embodiment of the invention, the liposome-magnetic articles can be targeted through the use of recognition elements, as discussed in this disclosure. Preferred targeting elements are those that promote cancer cell uptake. Most preferably, the recognition elements promote accumulation in the cytoplasm of cancer cells.

In another embodiment of the invention, the lipid-magnetic article structure may be stabilized through the crosslinking of the surface of the liposome. This may be accomplished through the incorporation of functionalized PEG-lipid molecules, for instance DSPE-PEG(2000)Maleimide, into liposomes in excess of that used for magnetic article attachment. The excess, along with maleimide or haloacetal groups on the magnetite articles attached to the liposomes, can be reacted with multifunctional PEG or carbohydrate, for instance PEG multithiol, such as PEG(5000)tetrathiol, or dextran multithiol, followed by capping of residual free thiols, for instance with a monofunctional maleimide compound, such as N-ethylmaleimide.

In another embodiment of the invention, the magnetic colloids are incorporated into the core of the bioactive agent-loaded liposomes.

The articles of the invention can be administered by injection (subcutaneous, intravenous, intramuscular, intradermal, intraperitoneal, intracerebral, or parenteral), with intraveneous injection being a preferred route. The articles may also be suitable for nasal, pulmonary, vaginal, ocular delivery and oral administration. The articles may be suspended in a pharmaceutically acceptable carrier for administration.

Reagents and starting materials in some embodiments can be obtained commercially from chemical distributors such as Sigma-Aldrich (St Louise, MO and Milwaukee, WI), Kodak (Rochester, NY), Fisher (Pittsburgh, PA), Pierce Chemical Company (Rockford, IL), Carbomer Inc. (Westborough, MA), Radcure (Smyrna, GA), and Polysciences (Niles, IL). PEG compounds may be purchased through companies such as NOF America Corporation (White Plains, NY), and Nektar (Birmingham, AL). Lipid and PEG-lipid structures can be obtained through Avanti Polar Lipids, Inc. and Northern Lipids, Inc.. Peptides to be used as REs can be purchased from many sources, one being Bachem (King of Prussia, PA). Proteins may be obtained from sources such as Calbiochem (San Diego, CA).

The following non-limiting examples are provided to further describe how the invention may be practiced.

EXAMPLES

Example 1. Preparation of Silica-Coated Magnetite Nanoparticle by Microemulsion

A stock solution of 1M Fe(II) was prepared by dissolving 0.278g $FeSO_47H_2O$ in 1mL of nitrogen-purged deionized water. Similarly, a stock solution of 1.5M Fe(III) was prepared by dissolving 0.4055g $FeCl_36H_2O$ in 1mL of nitrogen-purged deionized water. An oil phase solution containing cyclohexane, Igepal CO-210 and Igepal CO-720 surfactants was prepared with a weight percent of 83.35%, 7.3% and 9.35%, respectively. In a glass container, 25μL of the 1M Fe(II) solution and 25μL of the 1.5M Fe(III) solution were added to a 5mL solution of the oil phase under a nitrogen atmosphere, and the mixture was magnetically stirred for 1 hr to form a microemulsion. In another container, 100μL of NH_4OH (28-30 wt%) was added to a 5mL solution of the oil phase, and the mixture was magnetically stirred for 1 hr to form a NH_4OH microemulsion. In the absence of a magnetic field, the NH_4OH microemulsion was added dropwise to the Fe microemulsion with vigorous mechanical stirred for 1 hr to form magnetite nanoparticles.

50μL of tetraethylorthosilicate (TEOS) was then added to the magnetite nanoarticles solution and mechanically stirred for additional 24 hrs. Acetone was added

to the colloidal microemulsion to precipitate the silica-coated nanoarticles. The nanoarticles were washed with acetone and ethanol several times, then dissolved in water and lyophilized to obtain magnetite nanoparticles in powder form.

Example 2. Preparation of Inulin Magnetite Nanoparticle by Microemulsion: Coating Crosslinked by Free Radical Polymerization

A stock solution of 1M Fe(II) was prepared by dissolving 0.278g $FeSO_47H_2O$ in 1mL of nitrogen-purged deionized water. Similarly, a stock solution of 1.5M Fe(III) was prepared by dissolving 0.4055g $FeCl_36H2O$ in 1mL of nitrogen-purged deionized water. An oil phase solution containing cyclohexane, Igepal CO-210 and Igepal CO-720 surfactants was prepared with a weight percent of 83.35%, 7.3% and 9.35%, respectively. In a glass container, 25μL of the 1M Fe(II) solution and 25μL of the 1.5M Fe(III) solution were added to a 5mL solution of the oil phase under a nitrogen atmosphere, and the mixture was magnetically stirred for 30 minutes to form a Fe microemulsion. In another container, 100μL of NH_4OH (28-30 wt%) was added to a 5mL solution of the oil phase, and the mixture was magnetically stirred for 30 minutes to form NH_4OH microemulsion. In the absence of a magnetic field, the NH_4OH microemulsion was added dropwise to the Fe microemulsion with vigorous mechanical stirred for 1 hr to form magnetite nanoarticles.

166μL of monomers solution comprised of 25% Inulin Multi-Methacrylate (IMMA), 2% Cystine Bisacrylamide (CiBA) and 1% Sodium Acrylate (NaA) in 10mM Sodium Phosphate Buffer at pH7.2 was added to the magnetite nanoarticles solution, followed by 3μL of Sodium Persulfate (50mg/mL water) and 3μL of TEMED (5% solution). The solution was degassed using a water pump aspirator and mechanically stirred for 2 hrs to form a crosslinked scaffolding comprised of derivitized inulin. Ethanol was added to the microemulsion to precipitate the nanoarticles. The articles were then dissolved in water and purified using ion exchange BioBeads SM-2 for 2 hrs. The articles were filtered and lyophilized to obtain magnetite nanoarticles in powder form.

Example 3: Incorporation of Doxorubicin and Recognition Elements

Reduction of CiBA Containing Nanoarticles: Dissolve 1.0 g nanoarticles as prepared in Example 2 in 6.16 mL PBS at 65 mg/mL. Add 268 mg of DTT (FW 154; 24-fold excess per mole of CiBA) into the nanoarticle solution. Agitate the reaction for 2 hours at room temperature. Pass the nanoarticle solution through three FPLC desalting columns to remove excess DTT, using PBS as buffer. Collect the nanoarticle fractions and concentrate to a total of 10 mL of buffer (50 mg/mL of nanoarticles) using Amicon Ultra-15, MWCO 50k centrifugal filters (2.5 mL per filter) spun at 4000 rpm.

Linker Attachment: Add 233.6 mg of $PEG_{400}DBA$ (FW 641.86; 2.5-fold excess per mole of thiol) to the nanoarticle solution. Add 81.6 mg N-(ε-Maleimidocaproic acid) hydrazide (EMCH) (FW 225.24; 2.5-fold excess per mole of thiol) 5 minutes after $PEG_{400}DBA$ addition the nanoarticle solution. Agitate the reaction for 2 hrs after this step. Remove unreacted linkers by centrifuging in Amicon Ultra-15, MWCO 50K centrifugal filters spun at 4000 rpm, until about 250 μL remain. Reconstitute the nanoarticle retentate in 2.5 mL of 0.1 M PBS, pH 7.2 and repeat the centrifugation. Re-dissolve the retentate in each tube in 2.5 mL of 0.1 M PBS, pH 7.2.

Ligand Attachment: Add 42.08 mg of the peptide RGDdFC (FW 578; 0.5 equivalent of thiol) to solution 1 (any peptide or ligand with a free thiol, readily incorporated via a cysteine residue, can be attached to the nanoarticle through reaction with a PEG multibromoacetate linker).Take a 10 μL aliquot at t = 0 and t = 1hr after peptide addition for HPLC analysis. Agitate the reaction for 1 hr. Cap unreacted bromoacetamide with 17.6 mg of cysteine (FW 121.16; 1 equivalent of thiol) to each solution. Agitate the reaction for 10 minutes. Remove unreacted ligands by centrifuging each nanoarticle solution in Amicon Ultra-15, MWCO 50K centrifugal filters spun at 4000 rpm, until about 250 μL remain. Reconstitute each nanoarticle retentate in 2 mL of 0.1 M sodium phosphate buffer, pH 5, and repeat the centrifugation. Re-dissolve the retentate in 2.5 mL of 0.1 M sodium phosphate buffer, pH 5.

Doxorubicin attachment: Add 41.6 mg of doxorubicin (FW 579.99; 1 theoretical equivalent per 2 moles of thiol) to each of the two nanoarticle solutions (at 50 mg NP/mL (5 mL buffer)), first pre-dissolving doxorubicin in 8 mL of de-ionized water at 5.2 mg/mL. React at 37°C for 20 h. Remove unreacted doxorubicin using Amicon Ultra-15, MWCO

50K centrifugal filters spun at 4000 rpm, until about 250 μL remain. Reconstitute the nanoarticle retentate in 2.5 mL de-ionized water and repeat the centrifugation. Re-dissolve the retentate in 50 mL de-ionized water (5 mg/mL for lyophilization). Lyophilize the nanoarticle solution overnight.

Example 4:

Add 5 fold excess 5/8 arm-PEG_{2000}BA linker to reduced nanoarticles in 0.1M PBS, 1.2 g. Allow to react 2 hours at room temperature with agitation. Remove unreacted linker on FPLC with three 26/10 desalting columns in-line, equilibrated and run with 0.1M PBS pH 7.2.

Example 5: Preparation of Carbohydrate-Coated Magnetite Nanoparticle: Coating Crosslinked by Michael-type addition reaction

A stock solution of 1M Fe(II) was prepared by dissolving 0.278g $FeSO_47H_2O$ in 1mL of nitrogen-purged deionized water. Similarly, a stock solution of 1.5M Fe(III) was prepared by dissolving 0.4055g $FeCl_36H2O$ in 1mL of nitrogen-purged deionized water. An oil phase solution containing cyclohexane, Igepal CO-210 and Igepal CO-720 surfactants was prepared with a weight percent of 83.35%, 7.3% and 9.35%, respectively. In a glass container, 25μL of the 1M Fe(II) solution and 25μL of the 1.5M Fe(III) solution were added to a 5mL solution of the oil phase under a nitrogen atmosphere, and the mixture was magnetically stirred for 1 hr to form Fe microemulsion. In another container, 100μL of NH_4OH (28-30 wt%) was added to a 5mL solution of the oil phase, and the mixture was magnetically stirred for 1 hr to form NH_4OH microemulsion. In the absence of a magnetic field, the NH_4OH Microemulsion was added dropwise to the Fe Microemulsion with vigorous mechanical stirred for 1 hr to form magnetite nanoparticles.

150uL of 30% Inulin Multi-Methacrylate (IMMA) in 10mM Sodium Phosphate Buffer at pH7.2 was added to the magnetite nanoarticles solution, followed by 5.7μL of PEG_{3400}diThiol. The solution was mechanically stirred for 24 hrs. Ethanol was added to the microemulsion to precipitate the nanoparticles. The articles were then dissolved in

water and purify using ion exchange BioBeads SM-2 for 2 hrs. The articles were filtered and lyophilized to obtain magnetite nanoarticles in powder form.

Example 6: Attachment of Magnetite Article to Liposomes Comprised of Doxorubicin

50 mg DSPE–PEG(2000)Maleimide (Avanti Polar Lipids, Inc.) is added to a 10 mL Doxil solution containing 20 mg doxorubicin HCl. This solution is heated to 70 °C for 2 hours to incorporate DSPE –PEG(2000)Maleimide into the liposome.

1.0 g of magnetite material formed according to Example 2 is dissolved in 6.16 mL PBS at 65 mg/mL. 268 mg of DTT (FW 154; 24-fold excess per mole of CiBA) is added into the nanoarticle solution, and the reaction is agitated for 2 hours at room temperature. The resulting nanoarticle solution is passed through three FPLC desalting columns to remove excess DTT, using PBS as buffer. The nanoarticle fractions are collected and concentrated to a total of 10 mL of buffer (50 mg/mL of nanoarticles) using Amicon Ultra-15, MWCO 50k centrifugal filters (2.5 mL per filter) spun at 4000 rpm.

A portion of the reduced magnetite-containing nanoarticles are then reacted with the maleimide moiety, such that from one to ten magnetites are attached to each liposome. Add 1.0 g of 4-arm-PEG(5000)tetramaleimide (one maleimide moiety on the end of each of the four arms). Agitate the reaction for 2 hrs. Remove unreacted linker by centrifuging in Amicon Ultra-15, MWCO 50K centrifugal filters spun at 4000 rpm. Re-dissolve the retentate in 10 mL of 0.1 M PBS, pH 7.2.

Example 7:

Combine 1.0 mL of a 2.0 M $FeSO_4$ in 2 M HCl with 4.0 mL of a 1.0M $FeCl_3$ in 2 M HCl and stir with a magnetic stir bar. Add 50 mL of a 0.7 M NH_3 solution dropwise to the stirring solution. Allow the magnetite to settle and decant some of the liquid before centrifuging for 1 minute at 1000 rpm. Add 10 mL of 20% tetraethylammonium hydroxide to the precipitate and resuspend the magnetite. Use an aspirator vacuum to remove excess ammonia from the solution. Pour off some of the liquid and pour the magnetite-covered stir bar into a weigh boat. Using a strong magnet under the weigh boat to attract the magnetite, remove the stir bar and any excess liquid. Let the

magnetite dry over the weekend in the hood. Ferrofluid solution was prepared containing 100mg/mL of deionized water.

Aqueous monomers solution was prepared containing 30 wt% Inulin Multi-Methacrylate (IMMA), 4wt% Cystine Bisacrylamide (CiBA) and 2 wt% Sodium Acrylate (NaA) in deionized water. The oil phase was prepared containing cyclohexane, Igepal CO-720 and Igepal CO-210 with a ratio of 83.35, 9.35 and 7.3 wt%, respectively. Thermal initiator, sodium persulfate (NaPS), solution was prepared containing 250mg/mL of deionized water. N,N,N',N'-Tetramethylethylenediamine (TEMED) was used as purchased.

1mL of aqueous ferrofluid was mixed with 1mL of aqueous monomers. The combined solution was then added dropwise to the oil phase with mechanical stirring. 50uL of NaPS 250mg/mL solution and 12.5uL of TEMED were added to the microemulsion while stirring. The microemulsion was then transferred to a 100-mL Schlenk tube and degassed in an ice bath using a water pump aspirator. The degassed solution was placed on the shaker for 18 hrs. Ethanol was then added to the microemulsion to precipitate the nanoparticles. The particles were dissolved in water and purify using ion exchange (BioBeads SM-2) for 2 hrs. The particles were filtered and lyophilized to obtain monomer-coated magnetite nanoparticles.

Example 8:

A 0.27 M bis (2-ethylhexyl) sulfosuccinate sodium salt (Aerosol OT or AOT) solution was prepared by dissolving 12g AOT in 10mL isooctane. An aliquot of ultra-pure water was purged for one hour with N_2 gas. A stock solution of 1 M Fe(II) was prepared by dissolving 0.278g $FeSO_4.7H_2O$ in 1mL of the nitrogen purged water. Similarly, a stock solution of 1.5 M Fe(III) was prepared by dissolving 0.4055g $FeCl_3.6\ H_2O$ in 1mL of the nitrogen purged water. In a glass container, 25uL of the 1 M Fe(II) solution and 25uL of the 1.5 M Fe(III) solution were added to a 5mL aliquot of the AOT solution under a nitrogen atmosphere, and the resulting Fe/AOT mixture was magnetically stirred for 1 hr to form a Fe/AOT solution. In another container, 100uL NH_4OH (28-30 wt %) was added to another 5mL aliquot of the AOT solution, and the resulting NH_4OH/AOT mixture was magnetically stirred for 1 hr to form a NH_4OH /AOT solution. In absence of magnetic

field, the NH_4OH/AOT solution was added dropwise to the Fe/AOT solution with vigorous mechanical stirring for 1 hr. 50uL of tetraethylorthosilicate (TEOS) was then added to the resulting brown solution and mechanical stirring was continued for an additional 24 hrs to give a magnetite microemulsion.

Aqueous monomers solution was prepared containing 25 wt% Inulin Multi-Methacrylate (IMMA), 2 wt% Cystine Bisacrylamide (CiBA) and 1 wt% Sodium Acrylate (NaA) in 10mM sodium phosphate pH 7.2 buffer. The oil phase was prepared containing cyclohexane, Igepal CO-720 and Igepal CO-210 with a ratio of 83.35, 9.35 and 7.3 wt%, respectively. Sodium persulfate (NaPS) thermal initiator solution was prepared containing 250mg/mL of deionized water. N,N,N',N'-Tetramethylethylenediamine (TEMED) was used as purchased.

In a glass container, 0.5mL of aqueous monomer solution was added dropwise to 10mL of oil phase while stirring. 12.5uL of NaPS 250mg/mL solution and 5uL of TEMED were added to the microemulsion while stirring. With the magnetic stirrer removed, the monomer microemulsion was mixed with the magnetite microemulsion. The combined microemulsion was transferred to a 100-mL Schlenk tube and degassed in an ice bath using a water pump aspirator. The degassed solution was placed on the shaker for 18 hrs. Acetone was used to precipitate the nanoparticles. The nanoparticles were washed with acetone and ethanol several times with each solvent, then dissolved in water and lyophilized to obtain monomer-coated magnetite nanoparticles.

Example 9: Attachment of 4,4'-azobis(4-cyanovaleric acid) (ACVA) to an amine-containing hydrogel

One gram of magnetic cored nanoarticles having a hydrogel composition of 25/2/1 IMMA/CiBA/APMA is dispersed in 50 mL of 0.2 M pH=7.5 HEPES buffer. N-hydroxysuccinimide (NHS, 0.109 g) and ACVA (0.133 g) are dissolved in a second 50 mL quantity of the same buffer. To the solution containing the NHS and ACVA is added 1-[3-(dimethylamino)propyl]-3-ethylcarbodiimide hydrochloride (EDC, 0.182 g). After stirring the NHS/ACVA/EDC solution at room temperature for 10 minutes with a mechanical stirrer, the nanoarticles are added over the course or 1 minute. One hour later, with the aqueous mixture being continuously agitated by a mechanical stirrer over that interval, a

second quantity of EDC (0.182 g) is added. One hour later, a third quantity of EDC (0.182 g) is added. After one more hour, the particles are isolated by centrifugation or magnetic separation. Excess reagents and side products are removed from the particles by three re-suspension/isolation cycles, and the nanoarticle products are isolated by lyophilization.

Example 10: TEMPO-functionalized inulin

The product (1.0 g) of the reaction of 4-hydroxy-2,2,6,6-tetramethyl-piperdinyloxy, free radical (4-hydroxy-TEMPO) with 1,1'-carbonyldiimidazole (CDI) is added to an anhydrous DMSO (25 mL) solution containing inulin (DP=20, 1.22 g). The resulting mixture is stirred under a nitrogen atmosphere for two days, and is then poured into toluene (400 mL) with rapid stirring. The precipitated product is dried under vacuum, dissolved in de-ionized water, and dialyzed in a 500 MW cutoff dialysis membrane against de-ionized water. The product is isolated after lyophilization.

Example 11: Preparation of magnetic nanoarticles

Combine 1.0 mL of a 2.0 M $FeSO_4$ in 2 M HCl with 4.0 ml of a 1.0M $FeCl_3$ in 2 M HCl and stir with a magnetic stir bar. Add 50 mL of a 0.7 M NH_3 solution dropwise to the stirring solution. Allow the magnetite to settle and decant some of the liquid before centrifuging for 1 minute at 1000 rpm. Add 10 mL of 20% tetraethylammonium hydroxide to the precipitate and re-suspend the magnetite. Use an aspirator vacuum to remove excess ammonia from the solution. Pour off some of the liquid and pour the magnetite-covered stir bar into a weigh boat. Using a strong magnet under the weigh boat to attract the magnetite, remove the stir bar and any excess liquid. Let the magnetite dry for two days in a fume hood.

Example 12: Synthesis of radical generating hydrogel coated magnetic nanoarticles

An oil phase is prepared containing cyclohexane, Igepal CO-720 and Igepal CO-210 with a ratio of 83.35, 9.35 and 7.3 wt%, respectively. An aqueous phase is prepared by adding TEMPO-functionalized inulin (0.491 g) from Example 10, poly(ethylene glycol)

diacrylate (formula weight 575, 0.100 g), sodium acrylate (0.022 g), and ACVA (0.162 g) to 2.4 mL de-ionized water. Two mL of the resulting solution is added to 100 mg of the dry magnetic nanoarticles from Example 11. After thorough mixing, the aqueous phase is then added dropwise to the oil phase with mechanical stirring. The microemulsion is transferred to a 100-mL Schlenk tube and degassed in an ice bath using a water pump aspirator. The degassed solution is placed on a shaker for 2 hrs while being exposed to UV light. Ethanol is added to the microemulsion to precipitate the nanoparticles. The particles are dissolved in water and purified using ion exchange (BioBeads SM-2) for 2 hrs. The nanoarticles are filtered and lyophilized to obtain magnetic nanoarticles.